Vasoactive intestinal peptide receptor activity in human fetal enterocytes

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Functional and specific receptors for vasoactive intestinal peptide (VIP) (determined by their capacity to bind ^{125}I-VIP and activate adenylate cyclase) and cyclic AMP-dependent phosphodiesterase activities were characterized in enterocytes of human fetal small intestine between 18 and 23 weeks of gestation. Half-maximal stimulation of the cyclase and inhibition of ^{125}I-VIP binding in membrane preparations were respectively observed at 1.4 and 5×10^{-10} M VIP. The peptides structurally related to VIP activated the cyclic AMP generating system at pharmacological doses (10^{-7} M and above) in the following order of potency: VIP > PHI > GRF > secretin. Other peptides or test substances, including GIP, pancreatic glucagon, somatostatin-14, gastrin, CCK, neurotensin, pancreatic polypeptide, PYY, substance P, histamine and isoproterenol are inactive in this system, while the ubiquitous adenylate cyclase activators NaF, forskolin and prostaglandins were effective. These results, combined with the appearance of intestinal VIP in nerve fibers at 8 weeks and with the morphological and enzymatic maturation at 9–12 weeks of the intestinal mucosa, indicate that this neuropeptide may regulate either the differentiation or function of enterocytes during the early development of human intestinal mucosa.

Vasoactive intestinal peptide *Receptor* *Adenylate cyclase* *Human fetal enterocyte*

1. INTRODUCTION

Vasoactive intestinal peptide (VIP) was originally isolated from the small intestine in hogs by Said and Mutt [1,2]. VIP might function as a neurotransmitter [3] and affect the behavior of small intestinal epithelial cells by stimulating net fluid and electrolyte secretion from enterocytes [4–6]. As in the case of several other target organs [7,8], the biological effects of VIP on the small intestinal mucosa are probably mediated via a cyclic AMP-dependent mechanism after activation of cell surface receptors [4,5,9,10].

In human fetuses, VIP was detected as early as 8–10 weeks of age in the small and large intestine [11,12], and located in nerve fibers in the myenteric plexus at the 12th week of fetal age [11]. As VIP immunoreactivity during the early development of the human intestine might be of biological significance, this study was designed to identify and characterize pharmacologically functional VIP receptors in enterocytes isolated from fetuses. We therefore measured ^{125}I-VIP binding to membranes prepared from human fetal enterocytes, and compared the abilities of VIP and other peptides – some of which were related to the structural family of VIP and secretin [2,13] – to inhibit ^{125}I-VIP binding and to increase adenylate cyclase activity in the same preparation.

2. EXPERIMENTAL

2.1. *Chemicals*

Highly purified natural porcine VIP, PHI (porcine peptide with N-terminal histidine and C-terminal isoleucine amide) and GIP (gastric inhibitory peptide) were purchased from Professor V. Mutt (GHI Laboratory, Stockholm, Sweden). Crystallized, highly purified porcine glucagon

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(batch 421306) was from Novo Research Institute (Bagsvaerd, Denmark). Synthetic porcine secretin and human pancreatic growth hormone releasing factor (hpGRF) 1–40 were respectively prepared by Professor E. Wünsch (Max Planck Institut für Peptidchemie, Martinsried, FRG) and by Dr J. Rivier (Salk Institute, San Diego, CA). Synthetic cyclic ovine somatostatin-14, sulfated [Leu]^{15} human gastrin I, the C-terminal fragment of cholecystokinin (CCK 26-33), bovine substance P and neurotensin were from Beckman (Switzerland). Synthetic porcine peptide with N-terminal tyrosine and C-terminal tyrosine (PYY) was purchased from Peninsula Laboratories (Belmont, CA) and crystallized bovine pancreatic peptide batch 103411) was from Bachem (Budendorf, Switzerland). Forskolin, sodium fluoride, isoproterenol hydrochloride, histamine dihydrochloride, prostaglandins E_1 (PGE_1), 3-isobutyl-1-methylxanthine (IBMX), snake venom *(Ophiophagus hannah),* dithiothreitol, phenylmethylsulfonyl fluoride (PMSF), GTP and pure ATP were from Sigma (St. Louis, MO). Creatine phosphate (A grade) was from Calbiochem and phosphocreatine kinase, from Boehringer. [^{3}H]cyclic AMP and Na^{125}I were from the Radiochemical Centre (Amersham, England).

2.2. *Human fetal intestine*

Fetal human small intestine was obtained just after legal or therapeutically-induced abortions with the informed consent of the mothers. Small intestines from 4 fetuses (18–23 weeks gestational age) were dissected out and divided into five sections of equal length. Small bowel segments were opened along the lumen with scissors, and washed 3 times with ice-cold Krebs Ringer phosphate buffer (pH 7.5) prior to cell isolation. Intestinal fragments were then incubated for 15 min at 4°C in a solution containing 2.5 mM EDTA and 0.25 M NaCl (pH 7.5), as previously described for the removal of epithelial cells from rat colon and small intestine [9]. Intestinal cells were obtained by gentle shaking for 10 sec twenty times by hand. After each period of shaking, the intestine was placed in 50 ml of fresh medium. The resulting preparation was centrifuged ($200 \times g$, 4°C) for 2 min, and washed three times at the same speed with 40 ml of the EDTA/NaCl solution. Fig. 1 shows the typical appearance of the cell preparation containing enterocytes included in the intestinal villi structures [14].

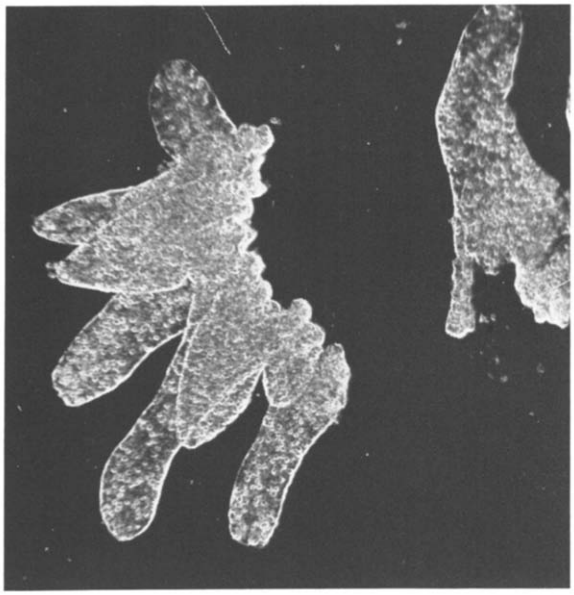

Fig.1. Morphology of small intestinal epithelial cells included in the intestinal villi in a human abortus at 18 weeks gestational age. Magnification, ×220.

2.3. *Membrane preparation*

Isolated human fetal enterocytes were disrupted at 4°C with a Virtis homogenizer (Cenco Instruments, Netherlands) in 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 30 mM NaCl, 1 mM dithiothreitol and 5 μM PMSF, using five bursts of 5 sec each. The broken cell suspension was then centrifuged ($600 \times g$, 10 min at 4°C) in a Sorvall centrifuge RC-2 (Newton, CT). Plasma membrane-enriched particles were obtained by differential centrifugation ($40\,000 \times g$, 20 min at 4°C). The membranes were stored frozen at −80°C for 1 week.

2.4. *Adenylate cyclase assay*

Adenylate cyclase activity was measured by a modified version of the method previously described [15]. The standard incubation mixture (final vol.: 250 μl) contained 1 mM ATP, 5 mM $MgCl_2$, the ATP-regenerating system (10 mM creatine phosphate and 0.5 mg/ml phosphocreatine kinase), 0.4 mM IBMX, 4 mg/ml BSA, 1 mM EGTA, 400 μg/ml bacitracin, 20 μM GTP and test substances

in 20 mM Tris–HCl (pH 7.5). The reaction was initiated by addition of the membrane-bound adenylate cyclase (5–10 μg membrane protein per tube) and the mixture incubated at 30°C for 15 min. Data are expressed as pmol cyclic AMP produced per min and per mg membrane protein.

2.5. *VIP binding assay*

The binding assay was conducted in a standard incubation solution containing in a final volume of 200 μl: 20 mM Tris–HCl (pH 7.5), 4 mg/ml BSA, 800 μg/ml bacitracin and ^{125}I-VIP at about 5×10^{-11} M. Reactions were started by adding the membrane suspension at a concentration of about 55 μg protein per 50 μl. Incubations were performed at 30°C for 60 min and stopped by filtration of a 150 μl aliquot through Millipore filters (EHWP 0.5 μm). Adsorption of ^{125}I-VIP to filters represented 0.7% of the total radioactivity added and 8% of the specific binding of ^{125}I-VIP to intestinal membrane, as determined by incubations with an excess (10^{-6} M) of native peptide. Results are expressed as the percentage of the radioactivity bound in the absence of unlabeled peptide (percent of maximum).

2.6. *Cyclic AMP-phosphodiesterase preparations and assay*

Freshly isolated enterocytes were sonicated in 40 mM Tris–HCl buffer (pH 8) containing 5 mM $MgCl_2$ and 3.8 mM β-mercaptoethanol. Phosphodiesterase activity [16] was measured in 250 μl reaction mixture containing 22 nM purified [^{3}H]cyclic AMP (120 000 cpm), 0.029–64 μM cyclic AMP, 50 μg snake venom, 25 μg cell protein, 5 mM $MgCl_2$, 3.8 mM β-mercaptoethanol in 40 mM Tris–HCl buffer (pH 8). The mixture was incubated at 30°C for 5 min. The reaction was stopped by addition of 500 μl anion-exchange resin (AGI-X2, 200–400 mesh) from Bio-Rad Laboratories, Richmond, CA. Data are expressed as pmol cyclic AMP hydrolyzed per min and per mg protein.

3. RESULTS

Adenylate cyclase activity in plasma membranes prepared from human fetal enterocytes was increased 4-fold by VIP and 2,5 and 20-fold by the ubiquitous activators PGE_1, sodium fluoride and forskolin, respectively (table 1). No effect on adenylate cyclase activity was exerted by the other peptides structurally related to VIP (glucagon, GIP, somatostatin-14) or by those related to neurotensin (pancreatic polypeptide and PYY), or gastrin (CCK). Substance P, histamine, or the beta adrenergic receptor agonist isoproterenol had no action on the membrane-bound adenylate cyclase either. We verified that reaction rates were linear with time (0–20 min) and with protein concentrations (4–35 μg membrane protein per tube) in the absence or presence of 10^{-7} M VIP or 5×10^{-3} M NaF (fig.2). In the adenylate cyclase assay, the

Table 1

Activation by peptides and ubiquitous activators of membrane-bound adenylate cyclase in human fetal enterocytes

Substance	Dose	*n*	cAMP (pmol/min per mg protein)
None	–	6	294 ± 31
VIP	10^{-7} M	6	1130 ± 68
PGE	4×10^{-4} M	1	588 ± 50
NaF[1]	5×10^{-3} M	6	1430 ± 100
Forskolin	4×10^{-5} M	3	5970 ± 551

Values are means ± SE from experiments performed on 4 different human fetuses between 18 and 23 weeks gestational age. The following peptides or test substances were found to be inactive in the same preparations: GIP (4×10^{-7} M); histamine (10^{-3} M); isoproterenol (2×10^{-6} M); pancreatic glucagon, somatostatin-14, gastrin, CCK, neurotensin, pancreatic polypeptide, PYY and substance P (10^{-6} M)

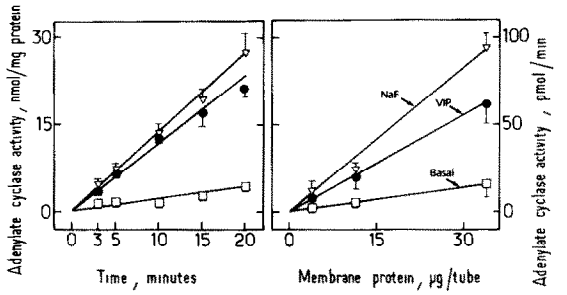

Fig.2. Effect of time (left) and membrane protein concentration (right) on adenylate cyclase activity in enterocytes isolated from human abortuses. No addition (□), 10^{-7} M VIP (●) or 5×10^{-3} M NaF (▽). Assay conditions were specified in section 2. Values are means ± SE from 4 experiments performed on 2 fetuses at 19 and 23 weeks gestation, respectively.

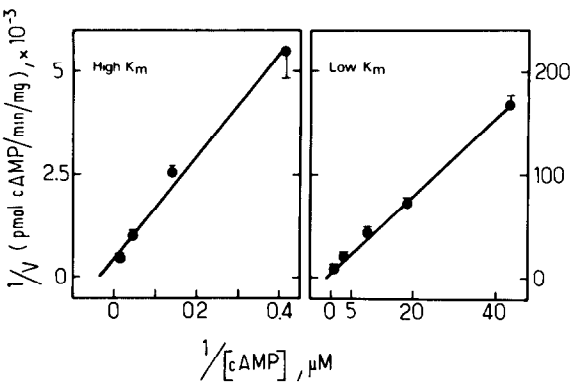

Fig.3. Lineweaver-Burk plots for cyclic AMP-phosphodiesterase activity in enterocyte homogenates from an 18 week-old human fetus. Apparent K_m and V_{max} values were determined by linear regression of the two linear components of the plot. The regression coefficients were respectively $r = 0.993$ ($p<0.01$) and $r = 0.999$ ($p<0.001$) for high (left) and low K_m phosphodiesterase (right). Incubation time and protein concentration were chosen so as to obtain the steady-state conditions for cyclic AMP hydrolysis. Results are means ± SE from 3 experiments performed in duplicate.

cyclic AMP phosphodiesterase inhibitor IBMX was added to the reaction mixture at a concentration of 0.4 mM in order to inhibit the cyclic AMP degrading enzymes. Kinetic analysis indicated two forms of cyclic AMP phosphodiesterase, one had a low K_m (1 ± 0.04 μM, $V_{max} = 130 \pm 10$ pmol cAMP/min per mg protein, as shown in fig.3, right) and the other a high K_m (35.2 ± 8 μM, $V_{max} = 2800 \pm 270$ pmol cAMP/min per mg protein, as shown in fig.3, left).

A significant increase in adenylate cyclase activity was detected with a VIP concentration as low as 10^{-12} M (fig.4, left). Half-maximal and maximal stimulations of this enzyme occurred with 1.4×10^{-10} M and 10^{-9} M VIP, respectively. PHI, hpGRF and secretin, three peptides structurally related to VIP (table 2), also raised adenylate cyclase activity in human fetal intestinal membranes, with a similar efficacy to that of VIP. However, VIP was about 10^3 times more potent than PHI ($K_a = 1.2 \times 10^{-7}$ M), 5.7×10^3 times more potent than hpGRF ($K_a = 8 \times 10^{-7}$ M) and 1.2×10^4 times more potent than secretin

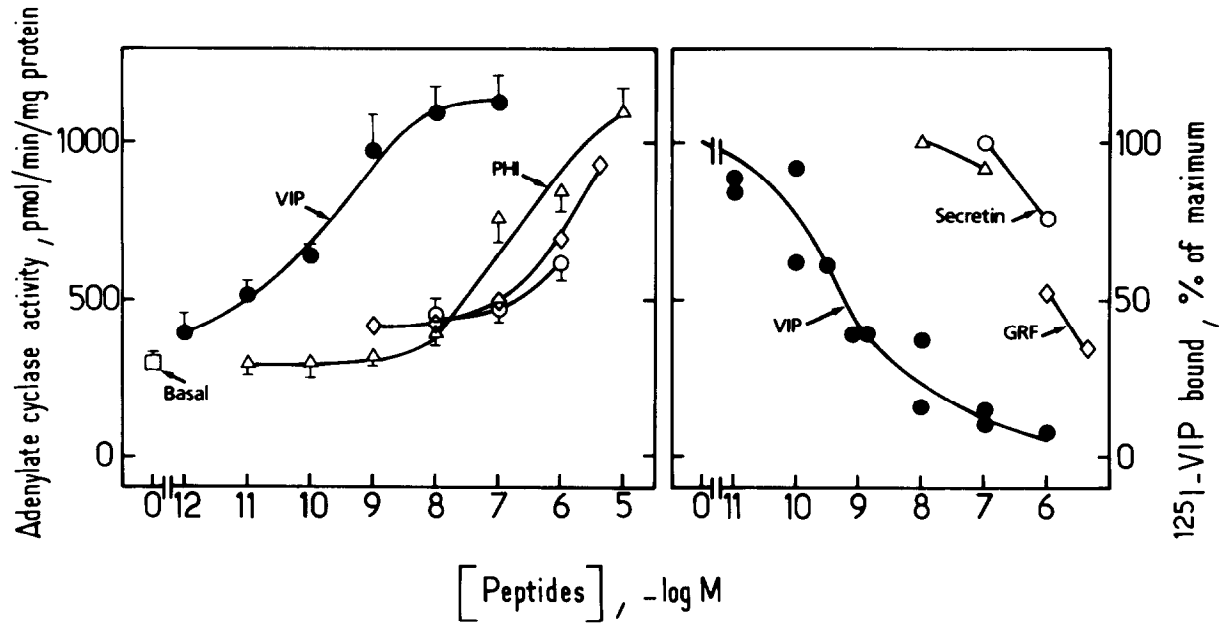

Fig. 4. Effect of VIP and peptides structurally related to VIP on adenylate cyclase activity (left) or ^{125}I-VIP binding (right) in membrane preparations from human fetal enterocytes. No addition (□), VIP (●), PHI (△), hpGRF (◇) or secretin (○). Data are means ± SE from 6 experiments performed in 2 fetuses of 19 and 23 weeks gestation, respectively (left). Individual determinations of membrane-associated ^{125}I-VIP radioactivity are represented on the right, from 2 experiments performed on one fetus at 23 weeks gestation. Results are expressed as the percentage of radioactivity associated with membranes incubated in the absence of unlabeled peptide (13 pmol ^{125}I-VIP/mg protein).

Table 2

Sequence homologies between the peptides of the secretin-VIP family

pVIP	HSDAVFTDNYTRLRKQMAVKKYLNSILN
pPHI	HADGVFTSDFSRLLGQLSAKKYLESLI
hpGRF	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL
pSecretin	HSDGTFTSELSRLRDSARLQRLLQGLV
pGlucaon	HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
pGIP	YAEGTFISDYSIAMDKIRQQDFVNWLLAQKGKKSDWKHNITQ
o Somatostatin	AGCKNFFWKTFTSC

Amino acids in identical positions are underlined: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophane; Y, tyrosine; p: porcine; hp: human pancreatic; o: ovine

($K_a = 17 \times 10^{-6}$ M). In agreement with these results, VIP, PHI, hpGRF and secretin were able to inhibit ^{125}I-VIP binding to plasma membranes prepared from human fetal enterocytes (fig. 4, right). VIP at a concentration of 5×10^{-10} M halved the initial binding (13 pmol ^{125}I-VIP/mg membrane protein), whereas with the highest peptide concentrations tested, binding was reduced by 9% (10^{-7} M PHI), 73% (4×10^{-6} M hpGRF) or 26% (10^{-6} M secretin).

4. DISCUSSION

We report here the presence of functional and specific receptors for VIP in enterocytes isolated from human fetuses at 18–23 weeks gestational age. The specificity of the receptors was determined by the use of peptides structurally related or unrelated to the VIP family (table 2). The relative potencies of these peptides for adenylate cyclase activation or ^{125}I-VIP binding (VIP > PHI > GRF > secretin) characterized the VIP recognition sites, since the same sequence was previously established in different tissues known to contain VIP receptors [7,9,10,17–22]. Such interactions of PHI, GRF and secretin with VIP receptors are due to the importance of the N-terminal region of the VIP molecule for its biological activity [23,24]. These four peptides possess 4–5 identical amino acid residues between positions 1 and 7 at their N-terminal end (table 2). In contrast, pancreatic glucagon, GIP, somatostatin-14 and other peptides or effectors unrelated to VIP were found here to be inactive in the adenylate cyclase system. Thus, the relative and absolute pharmacological specificity of the VIP receptor in human enterocytes are established at an early stage of fetal life and remain constant during development. Similarly, the activity and regulatory properties of the enzymes forming or degrading cyclic AMP were characterized in the present preparation. We also examined agents [25] that activate adenylate cyclase directly, via the catalytic subunit C (sodium fluoride, forskolin) or by interacting with the GTP-dependent regulatory complex N_i as well (forskolin).

Comparison of the potency of VIP in inhibiting ^{125}I-VIP binding and activating adenylate cyclase in human fetal enterocytes on the one hand and adult enterocytes on the other must be interpreted with caution, because of the differences in experimental conditions. For instance, in adults [19,20], experiments were performed on intact colonic crypts (to test ^{125}I-VIP binding) or on different membrane preparations and reaction mixture, to test adenylate cyclase activity. Nevertheless, it was observed here that for both these parameters VIP was about 4 times more potent in fetal than in adult enterocytes in man. In agreement with this observation we recently demonstrated [26] that rat fetal enterocytes at 19 days gestation are about 6 times more sensitive to VIP (EC_{50} = 2.5 × 10^{-10} M VIP) than adult rat enterocytes (EC_{50} = 15 × 10^{-10} M). Similarly, Laburthe et al. [20,27] have shown that human colonic HT-29 carcinoma cells are 4 times more sensitive to VIP (EC_{50} = 3 × 10^{-10} M) than colonic epithelial cells isolated from normal human colon (EC_{50} = 12 × 10^{-10} M).

This study provides the first example of VIP

receptor activity (determined by ^{125}I-VIP binding and adenylate cyclase activation) in human fetal tissue. The receptor-cyclic AMP system sensitive to VIP was functionally similar in fetal enterocytes and adult human intestine as regards pharmacological specificity, ^{125}I-VIP binding capacity, cyclic AMP-phosphodiesterase activity, and expression of the C and N_i components of adenylate cyclase, and perhaps more sensitive to VIP. These results confirm and extend those previously reported from this laboratory [26,28,29] indicating that specific receptors for the regulatory peptides of the VIP-secretin family are functional in the gastrointestinal mucosa during fetal life. In the human small intestine and duodenum, villi, crypts and specialized cell types appear at 9–12 weeks of gestation [14]. The outgrowth of villi in the duodenum at 8 weeks coincides with the maturation of the brush border enzymes sucrase, lactase and aminopeptidase in the small intestine [30]. If these results are considered together with previous histochemical and biochemical demonstrations of VIP immunoreactivity in nerve fibers at 12 weeks of fetal age [11,12], and with the early cellular differentiation and enzymatic maturation of the small intestinal mucosa [14,30], they indicate that VIP might have a direct effect on human fetal enterocytes and might also modulate their biological activities (metabolism, differentiation and function) from the fetal age in man. Studies are now in progress on cultured normal and cancerous intestinal cells in an attempt to confirm this hypothesis.

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